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Research article

Effect of gamma oryzanol-rich fraction from purple rice extract against lipopolysaccharide-induced vascular endothelial growth factor C production of human colon cancer cells and angiogenesis of human umbilical vein endothelial cells

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Abstract

Importance of the work: Bacterial infection can be a critical event following operative resection of colorectal cancer (CRC) and is known to be associated with CRC metastasis. Angiogenesis is a metastatic process and is mediated by several angiogenic factors within cancer cells—especially the vascular endothelial growth factor C (VEGFC)—and the angiogenic steps of endothelial cells. Lipopolysaccharide (LPS) from infecting bacteria acts as a strong trigger of angiogenesis.

Objective: To investigate the novel role of purple rice extract (PRE) on VEGFC production and the angiogenic process of LPS-stimulated colon cancer cells (SW480) and human umbilical vein endothelial cells (HUVECs).

Materials and Methods: Hexane soluble fraction (HSF) collected from PRE (identified as the γ-oryzanol (OR)-rich fraction) and purified OR were tested in LPS-induced SW480 cells and HUVECs. The proliferative ability was determined using colony formation assay and succinate dehydrogenase activity (MTT) assay, while the secretion and expression of VEGFC were investigated using enzyme-linked immunosorbent assay and western blot, respectively. Cell migration and tube formation were identified based on wound healing and tube formation assays.

Result: The inhibitory effect was confirmed of HSF on LPS responsiveness to VEGFC production in SW480, as well as in several angiogenic steps of HUVECs.

Main finding: The OR-rich fraction from PRE could be used as a further novel supplementary option, alongside conventional therapy, to mitigate the effects of infection and to minimize the adverse consequences of CRC resection.

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Introduction

Colorectal cancer (CRC) metastasis is the end stage of CRC, disseminating into distant organs, a serious event which is related to reduction of survival rate. Liver and lymph nodes are the most common CRC metastatic sites (Yin et al., 2020). Unfortunately, one of the major causes of CRC progression to this stage is bacterial infection during CRC resection. There is well established evidence supporting the relationship of this infectious complication to inflammation as well as CRC recurrence and metastasis (Kobayashi et al., 2007; van der Bij et al., 2009). The mechanism of these events is mainly regulated through the outer membranous part of bacteria via lipopolysaccharide (LPS) that binds with its specific receptors on remaining CRC cells, also known as the toll-like receptor 4 (TLR4), and this then activates several signal transductions. This LPS-TLR4 interaction mainly leads to inflammatory responses and CRC progression (Killeen et al., 2009; Hsu et al., 2011; Yesudhas et al., 2014; Zhu et al., 2016a).

Typically, cancer metastasis consists of several steps, including cancer cell over growth, reduced adhesion of cell to cell and cell to extracellular matrix (ECM), degradation of ECM proteins by proteases such as matrix metalloproteinase (MMPs), migration, invasion and angiogenesis, all of which may disseminate cancer cells to new distant sites (van Zijl et al., 2011). Angiogenesis, the formation of new blood vessels, is the main route of spread of cancer to distant organs. However lymphangiogenesis, the formation of new lymphatic vessels, also plays a role in cancer dissemination (Alitalo et al., 2005). This vessel formation is regulated by vascular endothelial growth factors (VEGFs) that are highly expressed in tumor cells. Among VEGFs, VEGFA is the main activator of angiogenesis, whereas VEGFC and VEGFD are essential for lymphatic vessel formation (Hicklin and Ellis, 2005). The expression of VEGFC in CRC is not only involved in tumor spread via lymphangiogenesis but is also correlated with metastasis and poor survival rates (Sleeman and Thiele, 2009; Zhu et al., 2016a). Interestingly, VEGFC is also an inducer of pathological angiogenesis (Benest et al., 2008; Tammela et al., 2008; Nagai and Minami, 2015). Therefore, interruption of VEGFC production was focused on, since its related metastatic effects were also disrupted, which led to improved survival rates in CRC patients (Jin et al., 2012). Other studies found that VEGF inhibition could interfere with several angiogenic steps of endothelial cells (EC), including the decrease of EC activation, degradation of basement membrane, proliferation, migration, tube formation (vascular tube) and maturation (Griffioen and Molema, 2000; DeCicco-Skinner et al., 2014; Norton and Popel, 2016). Undoubtedly, LPS works as a strong stimulator of VEGFC production in human colon SW480 cells as well as in the angiogenesis of human umbilical vein endothelial cells (HUVECs) (Zhu et al., 2016a; Li et al., 2017). Thus, a substance that can inhibit LPS-induced angiogenesis would be worthy of study. At this time, there is only quite limited evidence relating to the inhibitory effect of such substances on LPS-induced CRC metastasis (Rakhesh et al., 2012; Ying et al., 2018). A recent publication confirmed that the three main steps of CRC metastasis of LPS-induced human colon cancer SW480 cells (adhesion of cells to ECM, invasion and MMP-2 production) were inhibited by whole grain purple rice extract (Panyathep and Chewonarin, 2020). Although its inhibitory actions on many metastatic steps were revealed, the processes related to the angiogenesis step were not studied.

Purple rice (*Oryza sativa* L.) is a colored plant which is of increasing interest as part of health promotion; however, its therapeutic properties, such as anti-inflammation and anti-angiogenesis, have mostly only been studied in the rice bran part, (Tanaka et al., 2012; Sirithunyalug et al., 2018). In addition, its major components are divided into the hydrophilic part (rich in anthocyanins) and the lipophilic part (plenty in gamma oryzanol; OR), with many reports that anthocyanins possess anti-VEGF production in various cells (Oak et al., 2006; Wang et al., 2009) as well as anti-angiogenesis in HUVECs (Tanaka et al., 2012). In contrast, there is less information about anti-VEGF expression of OR in CRC (Kim et al., 2012) and its anti-angiogenesis in EC (Sakai et al., 2012). The current study aimed to completely investigate the inhibitory effect of the OR-rich fraction from purple rice extract on LPS-induced metastasis and to study the effect of this fraction, and OR, on LPS-induced VEGFC production in CRC and angiogenesis of EC.

Materials and Methods

Plant material and extraction

The extraction protocol was the same as Panyathep and Chewonarin (2020). Briefly, the powder of dried whole grains of purple rice (*Oryza sativa* L. var. *glutinosa*) was dissolved with 80% ethanol (ratio 1:5, weight per volume, w/v) and then was passed through Whatman filter paper no.1 to collect the filtrate. Thereafter, the filtrate was dried in an evaporator and lyophilizer to obtain crude ethanolic extract (CEE). Following

this, the CEE was fractionated, using hexane (a non-polar solvent) to separate the interesting lipophilic compounds into two main fractions: a hexane soluble fraction (HSF) and a hexane insoluble fraction (HIF). Then, HSF was selected as the main fraction of interest, since HSF has already been proved, using HPLC, to contain plenty of lipophilic γ-oryzanol (OR) and α-tocopherol (approximately 2,629.17 and 20.62 mg/100 g of HSF, respectively), and exhibited a strong inhibitory effect to LPS responsiveness in several metastatic steps of CRC (Panyathep and Chewonarin, 2020). Therefore, HSF and its major compound (OR) were also used in the current study. The OR was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

As mentioned previously, the metastatic ability of human colon cancer cells, derived from primary tumors (SW480 cells) is stronger than from their lymph node metastatic cells (SW620 cells) (Kubens and Zanker, 1998; Thuringer et al., 2015). Therefore, human colon cancer SW480 cells were used together with human umbilical vein endothelial cells (HUVECs), chosen and purchased from ATCC (Rockville, MD, USA). Cells were grown in culture medium supplemented with 10% fetal bovine serum (Invitrogen Corporation; New York, NY, USA). The SW480 cells were grown in Dulbecco's modified eagle medium (DMEM) (Invitrogen Corporation; New York, NY, USA) whereas the HUVECs were grown in endothelial cell growth media (ATCC; Rockville, MD, USA) containing various growth factors. Cells were cultured in a 5% CO_2 incubator at 37 °C. Both groups of cells were pre-treated with LPS (Sigma Aldrich; St. Louis, MO, USA) at 1 µg/mL for 4 hr before any experiments. The final concentrations of HSF and OR in media had already been verified from another study and were known to be non-toxic to both human colon cancer SW480 and normal fibroblast cells (Panyathep and Chewonarin, 2020) at 25–75 µg/mL and 2–8 µg/mL, respectively. Additionally, these concentrations were related with known possession of several inhibitory effects, consisting of anti-invasion, adhesion, migration and inflammatory responses on LPS-stimulated SW480 cells, especially HSF at 75 µg/mL (composed of 1.97 µg/mL of OR) and OR at 8 µg/mL (Panyathep and Chewonarin, 2020; Panyathep et al., 2021). Even though low concentrations of OR rarely expressed these inhibitory effects, the current study maintained these conditions to support the synergistic effects of various compounds in the OR-rich-fraction form PRE on angiogenesis.

Colony formation assay

This method was used to evaluate the anchorage-independent proliferation of SW480 cells to form colonies in soft agar. Briefly, 2.5 mL of 0.5% (w/v) base agar in DMEM was placed into 60 mm dishes. Thereafter, top agar consisting of 0.35% (w/v) agar in DMEM, LPS-treated SW480 cells (at 1×10^5 cells/mL) and HSF or OR treatment at various concentrations were prepared. Top agar (2.5 mL) was added on base agar and then cultured at 37 °C for 10 d. The qualitative and quantitative analysis of colonies (composed of at least 50 cells), were examined under a (10×) inverted microscope (10 random fields/dish). The data were expressed as the means of the percentage of colony formation (or number of colonies) versus the LPS control, from three independent experiments.

Enzyme linked immunosorbent assay

The secretion of VEGFC from LPS-induced SW480 cells was evaluated using colorimetric enzyme-linked immunosorbent assay ELISA assay. In short, SW480 cells (at 1×10^5 cells/60 mm dish) were pre-treated with LPS (1 µg/mL) for 4 hr. Then, the LPS was removed and replaced with HSF or OR at various concentrations. After incubation at 37 °C for 24 hr, each conditioned medium was collected and the content of VEGFC measured based on standard assay procedure (Sigma Aldrich; St. Louis, MO, USA). The total protein content of each sample was quantified and compared with its VEGFC content to ensure the equality of each sample. The results were expressed as the means of the percentage of VEGFC secretion (in picograms per milliliter versus 1 mg of total protein content) compared with the LPS control, from three independent experiments.

Western blot

The VEGFC expression was examined using immunoblot assay. Cell lysate protein (20 µg) of each treated condition of HSF or OR on LPS-induced SW480 cells (as described above) was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS-T containing 5% skim milk powder and then probed with specific primary monoclonal anti-VEGFC and β-actin antibody (in a dilution 1:1000; Merck Millipore; Burlington, MA, USA) overnight at 4 °C. After the membranes had been washed three times with PBS-T, they were further incubated with

diluted horseradish peroxidase (HRP)-conjugated secondary antibody (in a dilution 1:1000) (Merck Millipore; Burlington, MA, USA) at room temperature for 1 hr. Finally, enhanced chemiluminescence (ECL) substrate (Bio-Rad® Laboratories; Berkeley, CA, USA) was used for visualization of protein bands on the membranes. In addition, beta-actin was used as a loading control for each sample.

Cell proliferation assay

Succinate dehydrogenase activity (MTT) assay was used to evaluate the cell proliferation of HUVECs. First, 100 µL of HUVECs (5×10^4 cells/mL) were seeded onto 96-well plates and incubated at 37 °C overnight. Then, the medium containing LPS (at 1 µg/mL) was replaced on the old medium and incubated for 4 hr. Thereafter, LPS was removed. HSF and OR at various concentrations were added and further cultured at 37 °C for 4 hr or 24 hr. Then, MTT solution (final concentration of 0.5 mg/mL) was added and incubated for 3 hr. The insoluble formazan crystals that were created were dissolved using dimethyl sulfoxide. Finally, the colorimetric value was detected using a microplate reader at 540 nm. The data were presented as the means of percentage of cell viability (versus the LPS control) from three independent experiments.

Migration assay

EC migration is one of the angiogenic steps that also responds to LPS stimulation. Thus, wound healing assay was used to determine this ability by measuring the different migratory distances. In short, HUVECs were plated in 24-well plate (6×10^4 cells/well) and cultured overnight. The medium was removed and replaced with LPS (1 µg/mL) for 4 hr. Thereafter, the LPS was removed and a reference line was created using a pipette tip. Media containing each concentration of HSF or OR was added. The gap distance was measured using an inverted microscope at 0 hr and 24 hr of incubation. The data were presented as the means of the percentage of cell migration versus the LPS control from three independent experiments.

Tube formation assay

This *in vitro* method is a mimic for the *in vivo* angiogenesis process and is commonly used to determine an angiogenic factor or inhibitor. LPS-pretreated HUVECs at 24×10^4 cells/mL were suspended with HSF or OR at various concentrations at an equal volume ratio (1:1). Then, the mixture (100 µL) was added into a Matrigel-coated 96-well plate and incubated for 4 hr at 37 °C. Tube (capillary)-like structures were formed and the length of the tube was measured using a Zen program, in an inverted microscope (10×) in 3 random fields/well (Carl Zeiss Microscope; Jena, Germany). The results were expressed as the means of the percentage of tube length compared to the LPS control.

Statistical analysis

All results are displayed as means ± SD of three or more independent experiments. Data analyses were performed according to one-way analysis of variance following by mean comparisons based on least significant difference (LSD). All analyses were facilitated by the SPSS program (SPSS Inc.; Chicago, IL, USA) and the significance of the tests was set at $p \leq 0.05$.

Results

Effects of hexane soluble fraction and γ-oryzanol on colony formation of lipopolysaccharide-induced SW480 cell

Colony formation assay was performed to determine the cell proliferation ability of HSF and OR on LPS-treated SW480 cells. Treated SW480 cells with LPS (at 1 µg/mL for 4 hr) were sufficient to increase colony formation, by approximately 24.7%. HSF and OR significantly inhibited LPS-induced colony formation in the SW480 cells. However, only HSF had an inhibitory effect in a dose-dependent manner, of approximately 22–39% (Fig. 1B).

Effects of hexane soluble fraction and γ-oryzanol on expression and secretion of vascular endothelial growth factor C of lipopolysaccharide-induced SW480 cells

Relationships of LPS and VEGFC have been reported, as well as their involvement in various steps of CRC metastasis, such as invasion, migration and lymphangiogenesis (Zhu et al., 2016a, b). Therefore, inhibition of the production of these mediators was the therapeutic target. LPS (at 1 µg/mL for 4 hr) significantly increased the expression and secretion of VEGFC in SW480 cells, by 20% and 70%, respectively. HSF treatment significantly abrogated LPS-induced expression and secretion of VEGFC in a dose dependent manner, especially HSF 75

μg/mL (22% and 39% inhibition, respectively). In contrast, OR had no significant inhibitory effect on LPS-induced VEGFC secretion (Fig. 2C). Furthermore, OR treatment significantly increased LPS-induced VEGFC expression at low concentrations (2 μg/mL and 4 μg/mL), as shown in Fig. 2B. Therefore, the usage of purified OR might increase LPS responsiveness to VEGFC production and lead to the reduced efficacy of CRC therapy.

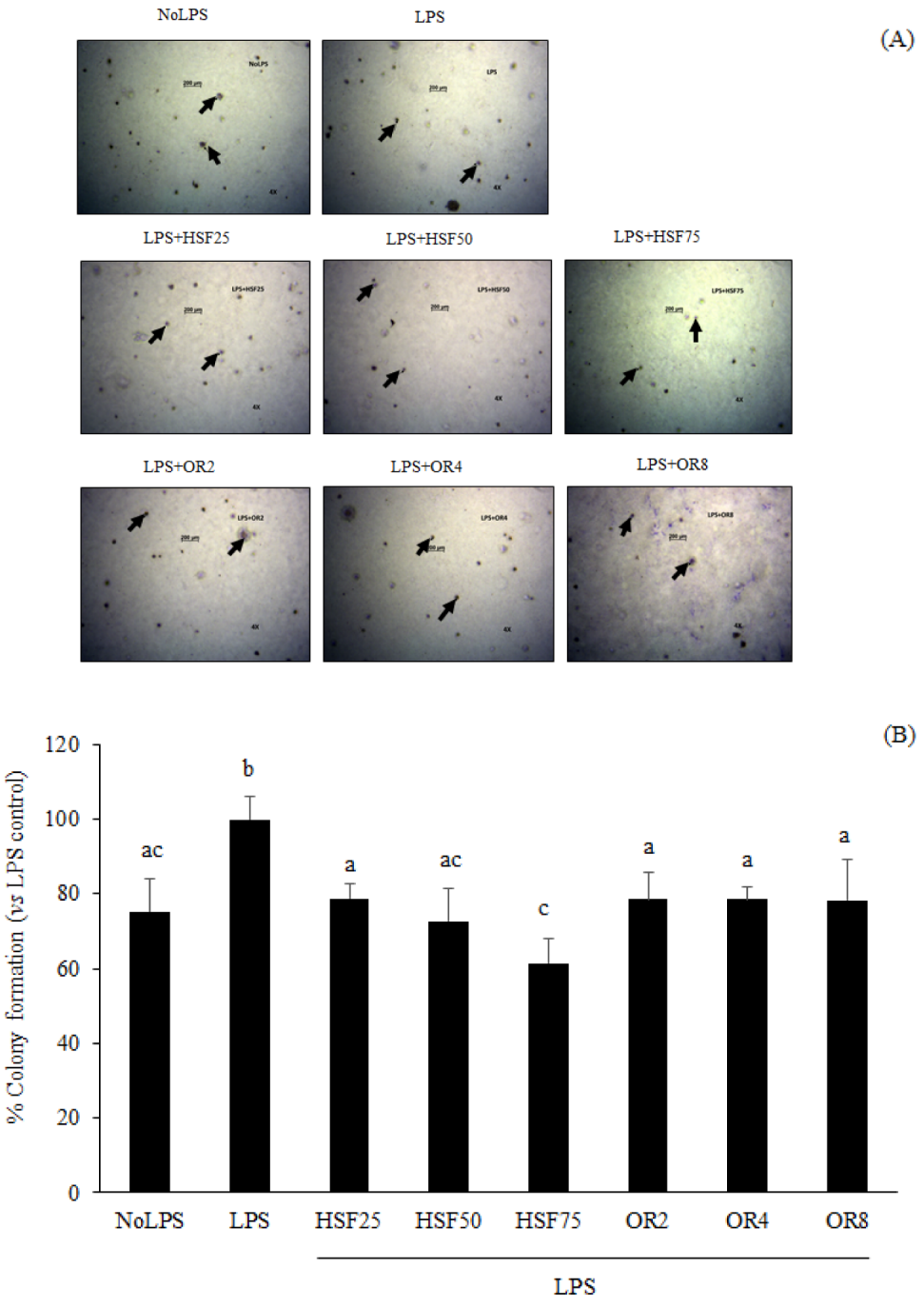

Fig. 1 Effects of hexane soluble fraction (HSF) and γ-oryzanol on colony formation of lipopolysaccharide (LPS)-induced SW480 cells: (A) images of colony formation at various conditions, where arrows indicate colony formation in soft agar (composed of at least 50 cells); (B) histograms showing mean % colony formation, where error bars indicate ± SD, $n = 3$, different lowercase letters above bars denote significant ($p \leq 0.05$) differences, HSF25, HSF50 and HSF75 are hexane-soluble fractions at 25 μg/mL, 50 μg/mL and 75 μg/mL, respectively, and OR2, OR4 and OR8 are gamma-oryzanol at 2 μg/mL, 4 μg/mL and 8 μg/mL, respectively

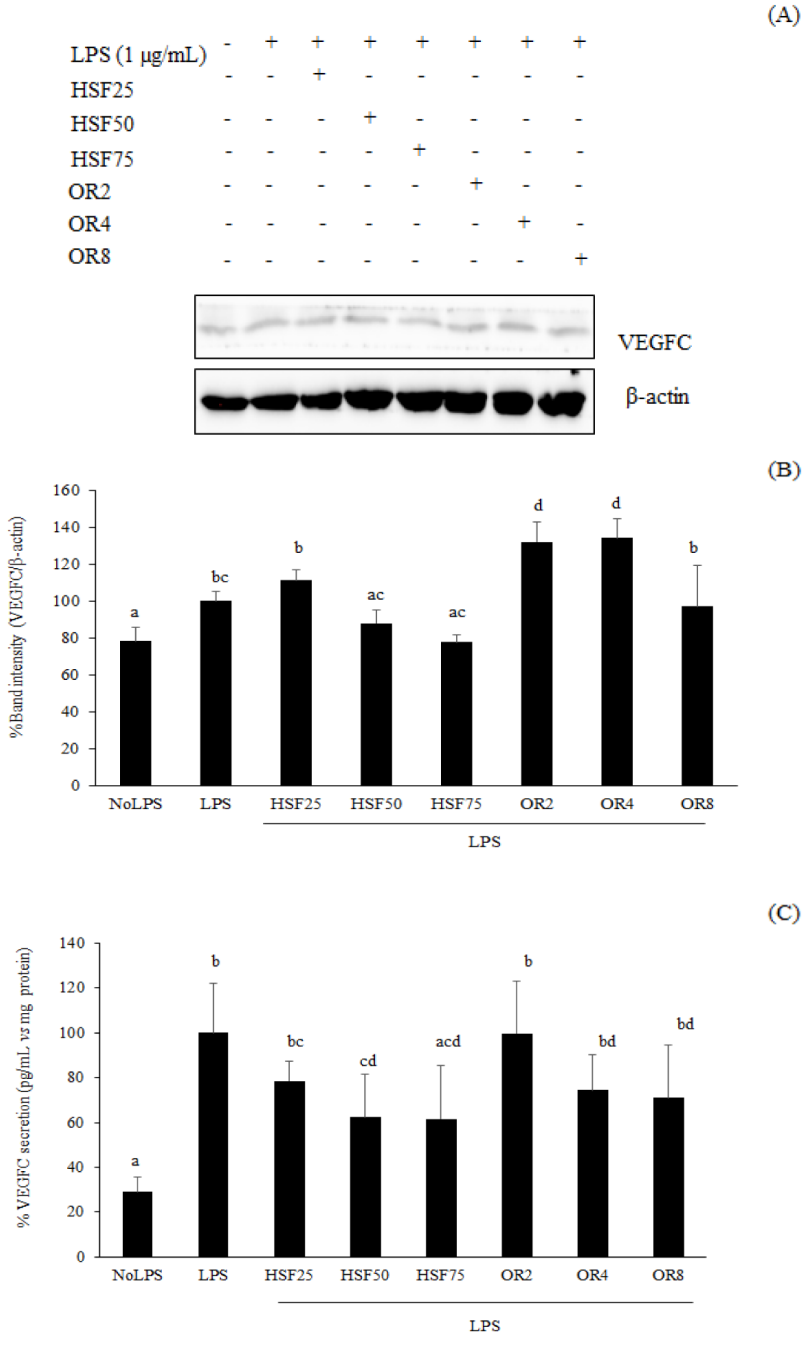

Fig. 2 Effects of hexane soluble fraction (HSF) and γ-oryzanol on vascular endothelial growth factor C (VEGFC) expression and secretion: (A) picture of VEGFC and β-actin bands; (B) histograms showing % band density (relative of VEGFC to β actin); (C) histograms showing % VEGFC secretion of lipopolysaccharide (LPS)-induced SW480 cells, where error bars indicate ± SD, $n = 3$, different lowercase letters above bars denote significant ($p \leq 0.05$) differences, HSF25, HSF50 and HSF75 are hexane-soluble fractions at 25 µg/mL, 50 µg/mL and 75 µg/mL, respectively, and OR2, OR4 and OR8 are gamma-oryzanol at 2 µg/mL, 4 µg/mL and 8 µg/mL, respectively

Effects of hexane soluble fraction and γ-oryzanol on cell proliferation of lipopolysaccharide-induced human umbilical vein endothelial cells

This experiment aimed to investigate the effect of HSF and OR on LPS-induced angiogenesis of HUVECs. EC proliferation is one important step in tumor angiogenesis, which was determined using MTT assay. The treated conditions in HUVECs were the same as in the SW480 cells. LPS pretreatment (at 1 µg/mL for 4 hr) significantly induced HUVEC proliferation for 24 hr (12% induction versus no LPS). HSF and OR strongly inhibited LPS-induced HUVEC proliferation, especially HSF at concentrations of 50 µg/mL and 75 µg/mL and at all OR concentrations, cell proliferation was decreased by more than 50%. These concentrations seemed to be toxic to HUVECs. However, HSF at a concentration of 25 µg/mL resulted in only 20%inhibition of HUVEC proliferation (Fig. 3). At this concentration, it was unlikely to be related to any cytotoxic effect on HUVECs.

Effects of hexane soluble fraction and γ-oryzanol on migrated ability of lipopolysaccharide-induced human umbilical vein endothelial cells

EC migration is one of the major critical steps of angiogenesis, which is also regulated by LPS (Li et al., 2017). The results from the wound healing assay demonstrated that LPS significantly promoted HUVEC migration (13% induction versus no LPS). This induction effect of LPS on HUVEC migration was abrogated by HSF and OR treatment (>50% inhibition). The result was correlated to previous cell proliferation assays (Fig. 3), which at high concentrations of HSF and at all concentrations of OR also dominantly displayed anti-LPS-induced EC migration. Only HSF at a concentration of 25 µg/mL could be interpreted as not causing a cytotoxic effect. HSF at 25 µg/mL significantly reduced HUVEC cell migration (53% inhibition), as shown in Fig. 4.

Effects of hexane soluble fraction and γ-oryzanol on tube formation of lipopolysaccharide-induced human umbilical vein endothelial cells

Tube formation assay is commonly used for evaluating factors involved in angiogenesis, with some studies showing that LPS (at 1 µg/mL) induced tube formation of EC (Zhu et al., 2016a; Li et al., 2017). In the current study, LPS (at 1 µg/mL for 4 hr) was active on tube formation by increasing tube length (31% induction). Even though, as shown in Fig. 5B, HSF and OR significantly reduced the length of tubes (*vs* LPS control), only the HSF action did so in a dose-dependent manner. To investigate the possible reason for the reduction in tube formation by HSF and OR, MTT assay was performed to test the effect of HSF and OR on HUVEC

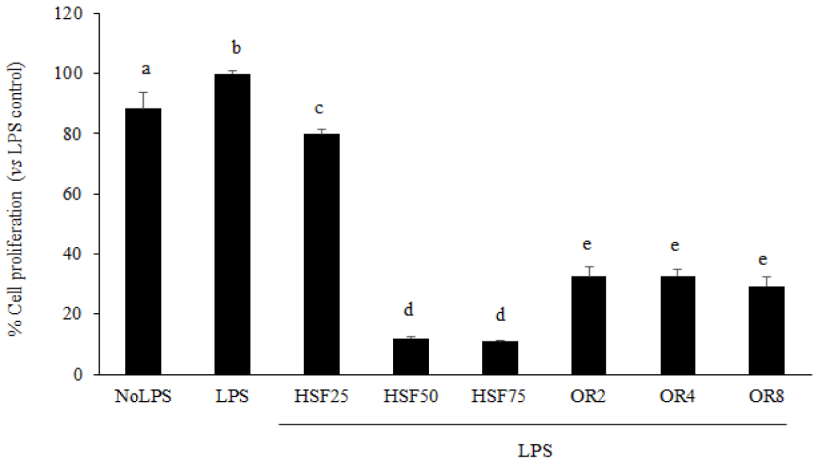

Fig. 3 Histograms showing effects of hexane soluble fraction (HSF) and γ-oryzanol on cell proliferation of lipopolysaccharide (LPS)-induced human umbilical vein endothelial cells represented by bar graph of percentage of cell proliferation (versus LPS control), where error bars indicate ± SD, $n = 3$, different lowercase letters above bars denote significant ($p \leq 0.05$) differences, HSF25, HSF50 and HSF75 are hexane-soluble fractions at 25 µg/mL, 50 µg/mL and 75 µg/mL, respectively, and OR2, OR4 and OR8 are gamma-oryzanol at 2 µg/mL, 4 µg/mL and 8 µg/mL, respectively

proliferation at 4 hr (the tube formation period). These MTT data (Fig. 5C) revealed that pretreatment with LPS (at 1 µg/mL for 4 hr) still had an induction effect on EC proliferation for 4 hr (11% induction versus no LPS) but that this effect was diminished by approximately 12–16% by HSF at 25 µg/mL and at all studied OR concentrations. However, HSF at 50 µg/mL and 75 µg/mL also restrained EC proliferation by approximately 35–39%. Thus, the attenuated action of HSF and OR on LPS-induced tube formation seemed to be related to cell proliferation. In other words, the inhibitory effect on LPS-induced cell proliferation of HSF and OR also contributed to the reduction of tube formation.

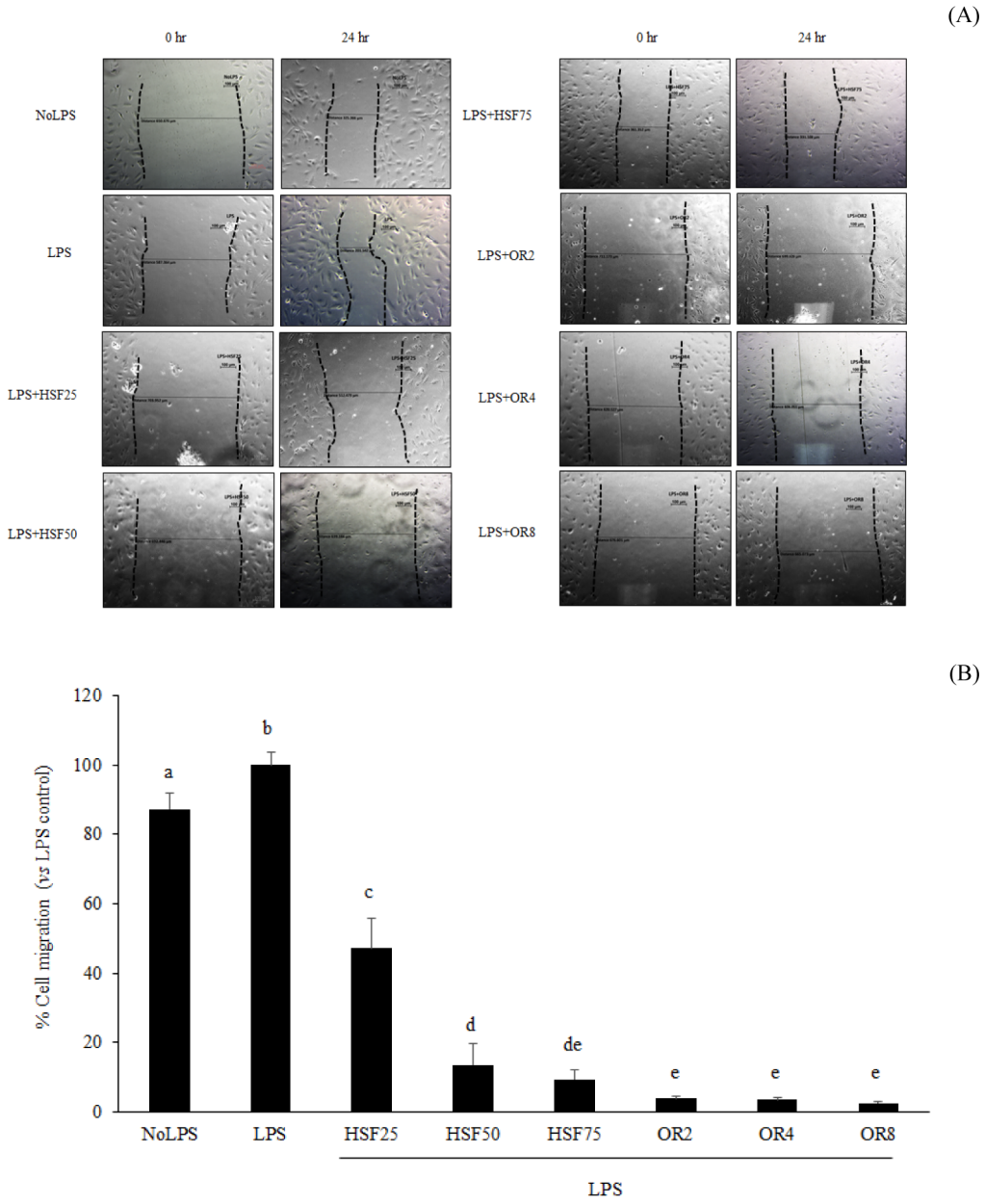

Fig. 4 Effects of hexane soluble fraction (HSF) and γ-oryzanol on migration of lipopolysaccharide (LPS)-induced human umbilical vein endothelial cells: (A) photographs of gap distance at 0 and 24 hr of treatment; (B) quantitative measurement, where error bars indicate ± SD, $n = 3$, different lowercase letters above bars denote significant ($p \leq 0.05$) differences, HSF25, HSF50 and HSF75 are hexane-soluble fractions at 25 µg/mL, 50 µg/mL and 75 µg/mL, respectively, and OR2, OR4 and OR8 are gamma-oryzanol at 2 µg/mL, 4 µg/mL and 8 µg/mL, respectively

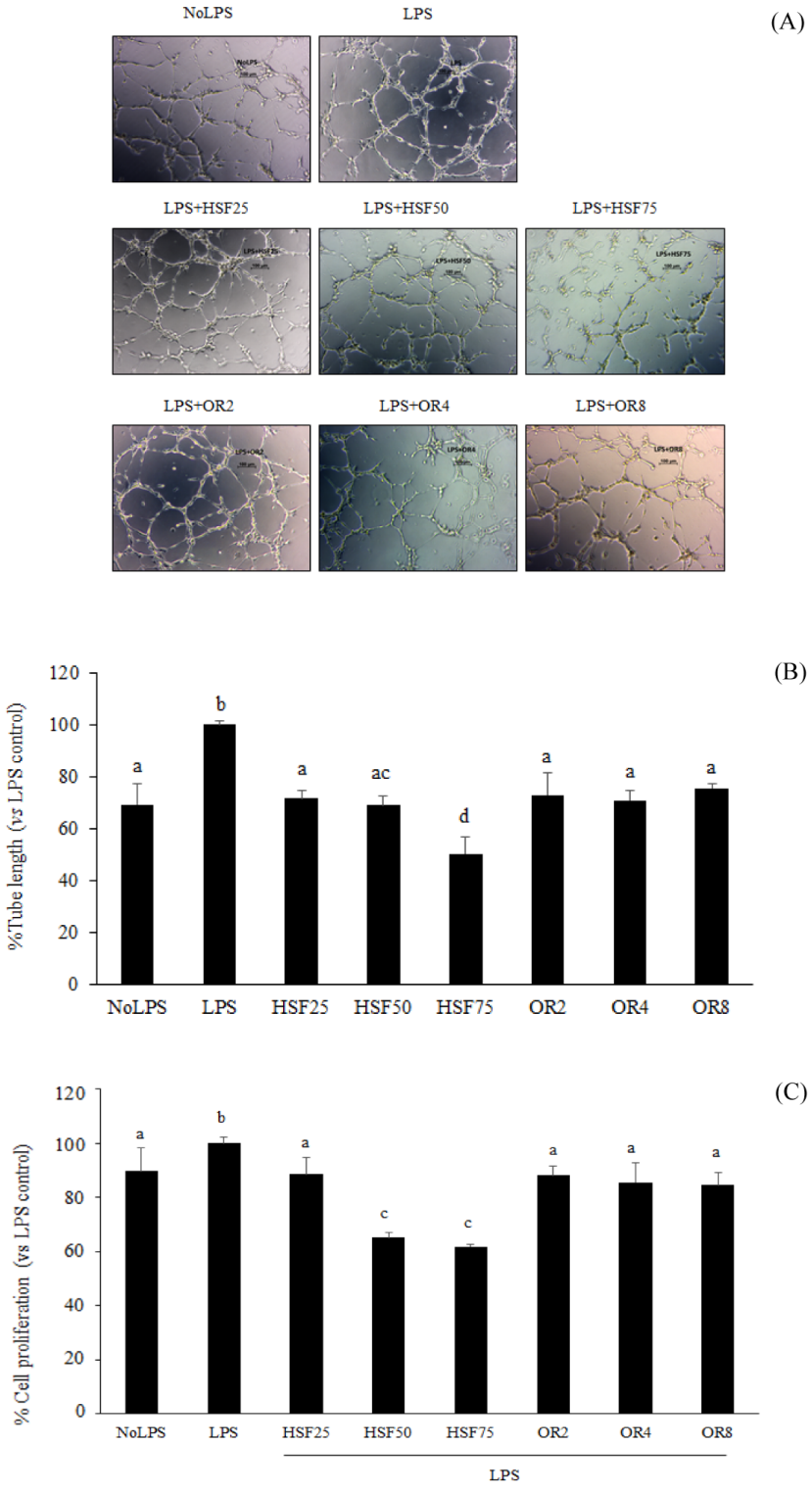

Fig. 5 Effects of hexane soluble fraction (HSF) and γ-oryzanol (OR) on tube formation of lipopolysaccharide (LPS)-induced SW480 cells. (A) pictures showing tube formation of each condition; (B) histograms showing % tube length; (C) histograms showing % cell proliferation of HSF or OR treated on LPS-pretreated human umbilical vein endothelial cells (at 4 hr), where error bars indicate ± SD, $n = 3$, different lowercase letters above bars denote significant ($p \leq 0.05$) differences, HSF25, HSF50 and HSF75 are hexane-soluble fractions at 25 µg/mL, 50 µg/mL and 75 µg/mL, respectively, and OR2, OR4 and OR8 are gamma-oryzanol at 2 µg/mL, 4 µg/mL and 8 µg/mL, respectively

Discussion

Infectious complication following CRC surgery is a critical event which leads to the progression of any remaining CRC to metastasis. As in another study, several steps of metastasis of colon cancer SW480 cells were increased after treating with LPS, such as adhesion, invasion and matrix metalloproteinase activity, which were then reduced in the presence of the OR-rich fraction (HSF) from PRE (Panyathep and Chewonarin, 2020). However, there was incomplete information about the protective effects of this compound on angiogenic processes, commonly regulated through various steps, including tumor over proliferation, release of angiogenic factors from tumor cells, EC activation, degradation of ECM to EC, EC proliferation, migration and tube formation. The current study aimed to complete the investigation of anti-LPS-induced metastasis of the OR-rich fraction, so the concentrations used in this study were kept the same as in the previous publication.

First, angiogenesis of CRC is controlled by the over proliferation of CRC. Once over the size of 1–2 mm^2, tumors need an increased nutrient and oxygen supply. This is achieved by creating new blood vessels from the existing vascular bed. Most data suggested that LPS itself had no effect on the proliferation of SW480 cells, based on various methods, such as MTT, CCK-18 and colony formation (Zhu et al., 2016a, b; Panyathep and Chewonarin, 2020). From the current data, LPS (at 1 µg/mL for 4 hr) was able to induce colony formation of SW480 cells (for 10 d). However, the previous study data showed that LPS had no effect on cell proliferation, based on MTT assay for 24 hr (Panyathep and Chewonarin, 2020). Additionally, using the same colony formation method, another study using longer LPS treatment periods of approximately 2 wk, produced a different result (Zhu et al., 2016a). Thus, using different methods and different incubation times for the LPS treatment, seemed to affect the results of SW480 proliferation. Consequently, there are no clear data about the possible effect of LPS on CRC proliferation, duration of the experiment, period of LPS treatment, sources of LPS (different species of bacteria) and dosage of the LPS treatment, which are most likely to be the important factors affecting CRC proliferation. However, even with or without LPS, HSF and OR expressed anti-proliferation action, as seen using SW480 cells (Panyathep and Chewonarin, 2020).

VEGFs are well known to be involved with angiogenesis. They are released from tumor cells, activating EC to create the new blood vessels. Of them all, VEGFA and VEGFC have been mostly found in CRC, however the major angiogenic factor is VEGFA (George et al., 2001). Recent data (not shown) already proved that LPS weakly induced VEGFA production in SW480 cells. VEGFC is more interesting, since supportive data revealed the LPS responsiveness of SW480 cells to VEGFC production. That data showed the correlation of LPS-induced VEGFC with the migration and invasive ability of CRC (Zhu et al., 2016a). VEGFC acts both as a potent lymphangiogenic factor and also contributes to angiogenic properties (Cao et al., 1998; de Oliveira et al., 2011). Furthermore, there was little information available related to substances that inhibit VEGFC production, so the current research aimed to primarily confirm the inhibitory action of PRE on LPS-induced VEGFC production in CRC. In line with other research, the current results indicated that LPS worked as a strong inducer of VEGFC production in SW480 cells. HSF performed anti-VEGFC expression and secretion. Noticeably, OR at low doses (2–4 µg/mL) slightly increased the action of LPS to VEGFC expression, which should be of concern when using this purified compound. This event seemed to be associated with not only the OR dose, but also several signaling molecules related to LPS-stimulated VEGFC expression on CRC, such as its specific receptor or VEGFR3 and TLR4-NF-κB/JNK pathways (Zhu et al., 2016a, b; Tacconi et al., 2019). Panyathep et al. (2021) reported that OR concentrations at 2 µg/mL and 4 µg/mL slightly increased p65 expression in LPS-stimulated SW480 cells, which might be the reason for the induction of VEGFC expression. However, other explanations may be revealed in future study. Thus, HSF would be the better option to use as an inhibitor of several adverse events from LPS.

In EC responsiveness, several steps of angiogenesis were induced by LPS stimulation, such as the ability of proliferation, migration and tube formation (Li et al., 2017). In the current study, LPS-induced HUVEC proliferation was strongly restrained by use of both HSF and OR. Even though almost all concentrations of HSF and OR used in the current study seemed to exhibit cytotoxicity to HUVECs (versus no LPS), this was not seen at the concentration of HSF at 25 µg/mL (its inhibitory effect was similar to the no-LPS condition). Additionally, these treated conditions have already been confirmed to have no cytotoxic effect on normal fibroblast cells (Panyathep and Chewonarin, 2020), together with another study that reported OR (30 µM or 18.1 µg/mL) had no effect on EC viability (Sakai et al., 2012). Thus, anti-LPS-induced EC proliferation of HSF at 50 µg/mL and 75 µg/mL (containing OR at 2.2 µM and 3.3 µM, respectively) as well as all OR treatments (2–8 µg/mL or 3.3–13.3 µM) might selectively respond to LPS-induced EC proliferation; however, the cytotoxicity of HSF to EC proliferation under no LPS-pretreated condition should be confirmed.

Furthermore, the anti-LPS-induced EC migratory patterns of HSF and OR were related to their anti-LPS-induced EC proliferation. As previously mentioned, both EC migration and proliferation were regulated by the adhesion of EC to specific ECM components (Davis and Senger, 2005). Furthermore, the actions of matrix metalloproteinases (MMPs) are also essential for breaking down adhesion proteins that are involved with cell-cell and cell-ECM, which also leads to cell migration (Chen and Parks, 2009). Specifically, MMP-2 and MT1-MMP appear to be associated with EC proliferation, migration, ECM adhesion and EC tube formation (Quintero-Fabian et al., 2019). Previously, OR (at 30 µM) was related to a decrease in the numbers of various adhesion molecules on EC, with and without LPS stimulation (Sakai et al., 2012). Additionally Panyathep and Chewonarin (2020) indicated that HSF (but not OR) was a potent inhibitor of LPS-induced MMP-2 activity in the supernatant of SW480 cells, with its zinc chelating activity. Thus, this was a reasonable explanation for the inhibitory actions of OR and the OR-rich fraction (HSF) on LPS-induced EC proliferation and migration. However, the effects of HSF and OR on LPS-induced EC adhesion should be further verified.

Finally, EC tube formation was determined using *in vitro* tube formation assay, which covered almost the whole process of angiogenesis (Gagnon et al., 2002) and specifically EC proliferation and migration, because they are important steps that take place before capillary-like structures are formed on Matrigel (basement membrane matrix) (DeCicco-Skinner et al., 2014). The results of tube formation were similar in the proliferation and migration experiments, which were enhanced by LPS and further suppressed by HSF and OR. The pattern of the HSF effect also occurred in a dose-dependent fashion. The trending of this inhibitory effect was correlated with anti-proliferation over the same time course (at 4 hr). Although the durations of the HSF and OR treatments for 4 hr were sufficient to reduce LPS-induced EC proliferation, this did not exceed 50% (the maximum was HSF 75 µg/mL, with only 40% inhibition). Correspondingly, anti-LPS induced EC proliferation seemed to contribute to anti-LPS-induced tube formation. At the same concentration of OR, the inhibitory effects of HSF at 75 µg/mL (containing OR 1.97 µg/mL) appeared to be stronger than for OR at 2 µg/mL. The synergistic effect of several compounds within HSF was most likely to be the main cause of these effects.

In conclusion, HSF (the OR-rich fraction from PRE), which contains various compounds, proved more interesting, due to its anti-LPS-induced metastasis actions and was more potent than purified OR. This research supported the therapeutic role of the OR-rich fraction in that it has anti-LPS induced metastatic potential, via attenuation of over-proliferation and VEGFC production in colon cancer cells, as well as in several angiogenic steps in EC, such as cell proliferation, migration and tube formation. Thus, this completed research on anti-LPS-induced metastasis of the OR-rich fraction confirmed the possible future usage of this extract as a supplementary treatment to CRC surgery that could reduce any adverse events related to post-operative bacterial infection. Additionally, it could increase the economic value of purple rice.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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